(FILE 'USPAT' ENTERED AT 14:07:05 ON 28 OCT 1998)

L1	811	S	FC(5A)RECEPTOR#
L2	549	S	L1(P)ANTIBOD?
L3	17	S	L2(P)BISPECIFIC
L4	71	S	L2(P)(ENHANCE OR INDUCE)
L5	6	S	L4(P)(IMMUNE(2A)RESPONSE)
L6	6	S	L5 NOT L3
L7	187	S	CD16
L8	116	S	L7(P)ANTIBOD?
L9	11	S	L8(P)BISPECIFIC
L10	2	S	L9 NOT L3
		E	RING, DAVID?/IN

5/7/2 (Item 2 from file: 5)
DIALOG(R)File 5:BIOSIS PREVIEWS(R)

09293717 BIOSIS NO.: 199497302087
Phase I trial of a **bispecific** murine monoclonal **antibody** targeting c-erbB-2 and **CD16**.

AUTHOR: Weiner L M(a); Ring R; Li W; Palazzo I E; Davey M; Rivera V; Alpaugh R K
AUTHOR ADDRESS: (a)Fox Chase Cancer Cent., Philadelphia, PA 19111, USA

JOURNAL: Proceedings of the American Association for Cancer Research Annual Meeting 35 (0):p219 1994

5/7/5 (Item 5 from file: 5)
DIALOG(R)File 5:BIOSIS PREVIEWS(R)

08763126 BIOSIS NO.: 199395052477
A human tumor xenograft model of therapy with a **bispecific** monoclonal **antibody** targeting c-erbB-2 and **CD16**.

AUTHOR: Weiner Louis M(a); Holmes Michele; Adams Gregory P; Lacreta Frank; Watts Perry; De Palazzo Irma Garcia
AUTHOR ADDRESS: (a)Dep. Med. Oncol., Fox Chase Cancer Center, 7701 Burholme Ave., Philadelphia, Pa. 19111

JOURNAL: Cancer Research 53 (1):p94-100 1993

ABSTRACT: New strategies are required to clinically exploit the ability of monoclonal antibodies to target tumor for lysis by cellular effector mechanisms. In this report we examine the therapeutic effects of 2B1, a bispecific monoclonal antibody with specificity for the extracellular domain of the c-erbB-2 oncogene product and the human Fc-gamma receptor, Fc-gamma-RIII (CD16), describe the characteristics and limitations of this model, and examine the mechanisms underlying the observed responses. The model uses SK-OV-3 human ovarian carcinoma xenografts in scid mice. These cells are susceptible to 2B1-directed lysis by human peripheral blood lymphocytes or lymphokine-activated killer cells, and maintain c-erbB-2 expression in vivo, 125I-labeled 2B1 selectively accumulates in tumor, with a peak of 10.5% injected dose/g of tumor 24 h following its i.v. injection. However, the selectivity of this binding is lessened by 2B1 accumulation in the lungs and other normal organs and persistence in the blood. This is caused by antibody binding to murine lung, colon, stomach, and skin expressing the epitope recognized by the anti-c-erbB-2 component of 2B1 in tumor-bearing, but not normal mice. In treatment studies using various permutations of antibody, human peripheral blood lymphocytes or lymphokine-activated killer cells and interleukin 2, cellular therapy alone had minimal effects on SK-OV-3 xenograft growth, but significantly improved when 2B1 treatment was incorporated. Median survivals increased from 80 +- 3.5 days with no therapy to 131 +- 7.3 days following therapy with 100 mu-g 2B1, interleukin 2, and human peripheral blood lymphocytes, with 70% of animals exhibiting no evidence of tumor at day 150. These effects were preserved when the cells were administered in human serum. In contrast, human serum abolished the antitumor effects of 520C9, which is the parent anti-c-erbB-2 antibody of 2B1. Thus 2B1-based therapy has therapeutic effects, without obvious

toxicity, despite [redacted] targeting of this antibody to [redacted]mal murine tissues. Since co[redacted]ation of 2B1 and interleukin 2 [redacted] have antitumor properties, mechan[redacted]ms other than **bispecific** monoclonal **antibody**-promoted conjugation of c-erbB-2 antigen-expressing tumor to **CD16**-expressing effector cells may be involved.

5/7/6 (Item 6 from file: 5)
DIALOG(R)File 5:BIOSIS PREVIEWS(R)

08717453 BIOSIS NO.: 199395006804
Antitumor effects of a **bispecific antibody** targeting CA19-9 antigen and **CD16**.

AUTHOR: De Palazzo Irma Garcia; Holmes Michele; Gercel-Taylor Cicek; Weiner Louis M(a)
AUTHOR ADDRESS: (a)Fox Chase Cancer Cent., 7701 Burholme Avenue, Philadelphia, Pa. 19111, USA

JOURNAL: Cancer Research 52 (20):p5713-5719 1992

ABSTRACT: **Bispecific** murine monoclonal **antibodies** that target tumor and Fc-gamma-RIII (**CD16**) can promote relevant tumor lysis by large granular lymphocytes. For these **antibodies** to be clinically useful, their properties should be maintained in vivo, where competing human immunoglobulin, shed target antigen, and shed **CD16** may be encountered. At a minimum, **bispecific antibody** antitumor effects should be preserved in whole blood. Furthermore, potentiation of tumor lysis should be reflected by demonstrating the ability of bispecific antibody-retargeted effector cells to infiltrate and mediate lysis of organized tumor. If these characteristics are demonstrated, and there is evidence of in vivo efficacy of bispecific antibody-based therapy in a relevant animal model, further clinical development of such antibodies would be warranted. In this report the ability of CL158 bispecific antibody supernatants to mediate lysis of SW948 tumor growing in monolayer is shown to be preserved in the presence of interleukin 2-activated whole blood. When SW948 cells were grown in vitro as multicellular human tumor spheroids, incubation with interleukin 2-activated lymphocytes (LAK cells) and CL158 led to structural and widespread necrosis. This was dependent on CL158 and resistant to competition by pooled human immunoglobulin or interleukin 2-exposed whole blood. These effects were not promoted by the monospecific antibodies produced by the parent clone of CD158 and were not observed when the IgG2a variant of CA19-9 antibody, which mediates conventional antibody-dependent cellular cytotoxicity, was used instead of its bispecific derivative. To examine the efficacy of bispecific antibody-based treatments on in vivo tumor, scid mice bearing early s.c. SW948 xenografts were treated with interleukin 2 for 5 consecutive days, supplemented by three i.v. injections of 10-7 human LAK cells and various antibodies. Treatment of mice bearing SW948 tumors with LAK cells did not retard tumor growth, but when CL158 was added, significant delays in tumor growth were observed. Tumor growth delay required treatment with both LAK cells and the bispecific antibody. Treatment with the IgGa variant of CA19-9 antibody, alone with LAK cells, had no effects on tumor growth. Although the mechanisms of these antitumor effects require further study, it is clear that human LAK cells treatment of animals bearing early, established s.c. tumors is enchanced by the addition of bispecific antibodies with relevant binding characteristics. When compared with the IgG2a isotype variant of CA19-9 monoclonal antibody, this bispecifically antibody offers the advantages of preservation of activity in physiological conditions, infiltration and disruption of organized tumor in vitro, and antitumor effects in a relevant xenograft model. These characteristics support the continued clinical development of bispecific antibodies with specificity for tumor and Fc-gamma-RIII.

5/7/10 (Item 10 from file: 5)
DIALOG(R)File 5:BIOSIS PREVIEWS(R)

07684055 BIOSIS NO.: 000092030976
BISPECIFIC MONOCLONAL **ANTIBODIES** DIRECTED TO **CD16** AND TO A TUMOR-ASSOCIATED ANTIGEN INDUCE TARGET-CELL LYSIS BY RESTING NK CELLS AND BY A SUBSET OF NK CLONES

AUTHOR: FERRINI S; PRIGIONE I; MIOTTI S; CICCONE E; CANTONI C; CHEN Q; COLNAGHI M I; MORETTA L
AUTHOR ADDRESS: IST. NAZIONALE PER LA RICERCA SUL CANCRO, V.LE BENEDETTO XV, 10, 16132 GENOA, ITALY.

JOURNAL: INT J CANCER 48 (2). 1991. 227-233.
FULL JOURNAL NAME: International Journal of Cancer

ABSTRACT: CD16 surface antigens represent activatory molecules in CD3-16+ NK cells. In order to target NK cells against relatively. NK-resistant ovarian carcinomas, we used an anti-CD16 monoclonal antibody (MAb) (VD4), together with an anti-ovarian carcinoma-associated antigen (MOV19), to construct biMAbs. To this end, hybrid hybridomas were generated by fusing a TK-deficient VD4 hybridoma mutant with a HGPRT-deficient MOV19 hybrid. Supernatants from hybrid hybridomas that had been selected in HAT medium were screened for their ability to induce a CD3-16+ NK clone to lyse an MOV19+ ovarian carcinoma cell line in a 4-hr 51Cr-release assay. The NMB.45 hybrid hybridoma secreted a biMAb which triggered lysis of MOV19+ but not of MOV19- target cells. Some degree of target cell lysis was also observed with MOV 19 MAb (due to ADCC mechanisms), while the VD4 MAb was ineffective. HPLC fractionation of MAbs secreted by the hybrid hybridoma made it possible to identify 4 different peaks, one of which appeared to contain functional biMAb molecules. HPLC-purified biMAb (100 ng/ml) induced resting PBL to lyse the "NK-resistant" IGROVI ovarian carcinoma cell line. Fresh MOV19+ tumor cells were also lysed, although with lower efficiency. When IL-2-activated lymphocytes were used as a source of effectors, biMAb caused only minor increases in the IL-2-induced cytolytic activity. Further analyses of the effect of biMAb were performed at the clonal level. Among CD3-16+ NK cell clones, a clear enhancing effect could be observed only in GL183+ but not in GLI183- clones. In CD3+ cytotoxic clones a triggering effect could be detected in one out of 4 TCR.gamma./.delta.+ clones but not in TCR .alpha./.beta.+ clones.

5/7/151 (Item 139 from file: 34)
DIALOG(R)File 34:SciSearch(R) Cited Ref Sci

02630601 Genuine Article#: LR050 Number of References: 0
(NO REFS KEYED)
Title: USE OF ANTI-CD3 AND ANTI-**CD16** **BISPECIFIC** MONOCLONAL-**ANTIBODIES** FOR THE TARGETING OF T-CELLS AND NK CELLS AGAINST TUMOR-CELLS
Author(s): FERRINI S; CAMBIAGGI A; SFORZINI S; CANEVARI S; MEZZANZANICA D; COLNAGHI MI; MORETTA L
Corporate Source: IST NAZL RIC CANC/I-16132 GENOA//ITALY/
Journal: CANCER DETECTION AND PREVENTION, **1993**, V17, N2, P295-300
ISSN: 0361-090X
Language: ENGLISH Document Type: ARTICLE
Abstract: To target T lymphocytes against EGF-R+ tumors, we constructed anti-CD3/anti-EGF-R bimAbs either by the generation of a hybrid hybridoma (quadroma) or by a chemical cross-linking method. Analysis of the in vitro functional activity of these two different constructs

indicated that th[redacted]uadroma-secreted bimAb was mor[redacted]fficient in targeting the CD[redacted] clones against EGF-R+ target [redacted]s with respect to the bimAb produce[redacted] by chemical method. In addition, the quadroma-produced bimAb is able to induce cytolysis of EGF-R+ tumor cell lines of PHA-induced lymphoblasts that had been expanded in IL-2-containing medium, whereas tumor cells lacking expression of EGF-R were not lysed. Resting PBL targeted by the bimAb did not display significant cytotoxicity against the relevant tumor.

An anti-CD16 hybridoma (IgG1) was fused with an anti-folate-binding protein hybrid (IgG2a) to construct bimAbs to target NK cells against NK-resistant ovarian carcinomas. The hybrid IgG1/IgG2a bimAb triggered the specific lysis of relevant target cells by resting NK cells, but it was ineffective when CD8+TCRalpha/beta+ cultured cell populations were used as effectors. Only marginal increases of cytolytic activity could be induced by the bimAb when IL-2-activated PBL (i.e., LAK cells) were used as effectors due to the high cytolytic activity of these cells against the relevant tumors in the absence of bimAb.

The possible use of anti-CD16 or anti-CD3 bimAbs for the development of different cellular immunotherapy strategies against cancer is discussed.

5/7/155 (Item 2 from file: 76)
DIALOG(R)File 76:Life Sciences Collection

01680645 2929768
Antitumor effects of a **bispecific antibody** targeting CA19-9 antigen and **CD16**.
Garcia de Palazzo, I.; Holmes, M.; Gercel Taylor, C.; Weiner, L.M.
Fox Chase Cancer Cent., 7701 Burholme Ave., Philadelphia, PA 19111, USA
CANCER RES. vol. 52, no. 20, pp. 5713-5719 (**1992**.)
DOCUMENT TYPE: Journal article LANGUAGE: ENGLISH
SUBFILE: Immunology Abstracts

Bispecific murine monoclonal **antibodies** that target tumor and Fc gamma RIII (**CD16**) can promote relevant tumor lysis by large granular lymphocytes. For these **antibodies** to be clinically useful, their properties should be maintained in vivo, where competing human immunoglobulin, shed target antigen, and shed **CD16** may be encountered. At a minimum, **bispecific antibody** antitumor effects should be preserved in whole blood. In this report the ability of CL158 **bispecific** antibody supernatants to mediate lysis of SW948 tumor growing in monolayer is shown to be preserved in the presence of interleukin 2-activated whole blood. When SW948 cells were grown in vitro as multicellular human tumor spheroids, incubation with interleukin 2-activated lymphocytes (LAK cells) and CL158 led to structural and widespread necrosis.

5/7/159 (Item 1 from file: 173)
DIALOG(R)File 173:Adis LMS Drug Alerts

00431358 800177591
TITLE: A human tumor xenograft model of therapy with a **bispecific** monoclonal **antibody** targeting c-erbB-2 and **CD16**.
AUTHOR: Weiner L M; Holmes M; Adams G P; et al
JOURNAL: Cancer Research (Cancer-Res) 53: 94-100, 1 Jan 1993.
PUBLICATION DATE: 1 January 1993 (**19930101**)
LANGUAGE: English
ADIS LMS: Cancer Chemotherapy (Index only): Alert no. 2, 1993

inducing natural killer cell (NKC)-dependent cytotoxicity. The process involves: co-culturing hybridoma cells producing anti-CD16 MAbs together with activated human NK cells; monitoring the mortality rate of the hybridoma cells; and selecting the hybridoma cells with the highest mortality rate. Also claimed is hybridoma cell line A9 (DSM ACC 2148) which produces a monoclonal antibody of subclass IgG1-lambda, is directed against human CD16 antigen, has a high capacity for inducing NKC-dependent cytotoxicity, and binds to a CD16 epitope separate from that recognized by MAb 3G8. Also claimed is a process for producing bispecific MAbs by fusing hybridomas cells producing anti-CD30 MAb to obtain a tetradoma, and isolating and purifying the resulting bispecific antibodies. The bispecific antibodies are useful for inducing NKC-mediated cytotoxicity against human tumor cells to cause regression of established tumors, especially Hodgkin disease. (14pp)

5/7/166 (Item 3 from file: 357)
DIALOG(R)File 357:Derwent Biotechnology Abs

0150619 DBA Accession No.: 93-08671
Methods useful for the generation of bispecific antibodies - **bispecific antibody** production and screening from a **CD16** Fab cDNA-transfected COS-1 cell culture (conference paper)
AUTHOR: Stanger E; Scheirer W; Pursch E; Peichl P
CORPORATE AFFILIATE: Sandoz
CORPORATE SOURCE: Institute of Applied Microbiology, Vienna, Austria.
JOURNAL: Anim.Cell Technol. (586-91) **1992**
LANGUAGE: English
ABSTRACT: A screening system was developed for quadroma (tetradoma) clones, to detect bispecific antibody (Ab) production and to compare the quadroma binding characteristics relative to the original Fab fragment. COS-1 cells (ATCC CRL-1650) were transfected with solution A (0.1 ml/sq cm 2 ml Dulbecco's modified Eagle's medium (DMEM) containing 10% NU serum, 10 mM HEPES, 50 ul DEAE dextran in phosphate buffered saline (PBS), 80 ul chloroquine in PBS and 1 ug CD16 cDNA). Subsequently the medium was removed from the transfected cells before adding the cell culture supernatant containing Abs. The Ab bound to the transfected COS-1 cells was visualized by FITC labeled anti mouse IgG for 30 min at 4 deg. After washing with 1% CSA in PBS the lids were directly screened using an epifluorescence microscope. The samples containing anti-CD16 Abs showed a clear membrane fluorescence on 10-30% of all cells (depending on the transfection rate). The modified ELISA technique affinity test allowed comparison of relative binding characteristics between Fab fragments and bispecific Abs. (3 ref)

5/7/167 (Item 4 from file: 357)
DIALOG(R)File 357:Derwent Biotechnology Abs

0141802 DBA Accession No.: 92-14294
Targeting cytotoxicity with a bi-specific antibody directed to c-erb-2 and human Fc-gamma receptor III - human oncoprotein human Fc-gamma receptor-III **CD16 bispecific antibody** production, purification; tetradoma 2B1 construction (conference paper)
AUTHOR: Ring D B
CORPORATE AFFILIATE: Cetus
CORPORATE SOURCE: Department of Immunology, Cetus Corporation, 1400 Fifty-Third Street, Emeryville, CA 94608, USA.
JOURNAL: Antibody Eng. (3 pp.) **1991**
CODEN: 9999X
LANGUAGE: English
ABSTRACT: Hybrid hybridoma 2B1 producing a bispecific antibody with binding sites for human oncogene product c-erbB-2 (aka HER2 or neu) and for

human Fc-gamma [redacted]tor-III (aka CD16) was pr[redacted]ed by fusion of hybridoma 520C9 ([redacted]e IgG1-kappa recognizing the o[redacted]roteins) and 3G8 (recognizing Fc-gamma receptor-III or **CD16**). The **bispecific antibody** was purified by DEAE-Sepharose, mono-Q-HPLC and SDS-PAGE anion-exchange chromatography in reasonable yield, since heterologous pairing of antibody light and heavy chains was not observed in this particular hybrid hybridoma. 2B1 bispecific antibody targeted human LGLs and cultured human monocytes to lyse a variety of c-erbB-2 positive human cancer cell lines. Cytological targeting was not diminished in 100% autologous human serum, and were observed in conditions designed to model an in vivo situation (e.g. in the presence of excess PMNs, in whole blood, or with effector and target cells from human cancer patients). Other bispecific antibodies formed from fusions of 3G8 are discussed, and include P-glycoprotein-specific and Fc-gamma

RECORD TYPE: Cita[redacted]n
DOCUMENT TYPE: Ani[redacted]

DESCRIPTORS: Biotechnology; Bispecific-monoclonal-antibody-2B1, pharmacodynamics; Ovarian-cancer; Research-and-development

5/7/160 (Item 2 from file: 173)
DIALOG(R)File 173:Adis LMS Drug Alerts
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00278854 800309915
TITLE: Phase I trial of 2B1, a **bispecific** murine monoclonal **antibody** targeting c-erbB-2 and **CD16**.
AUTHOR: Weiner L M; Ring D; Li W; et al
JOURNAL: Proceedings of the American Society of Clinical Oncology (Proc-Am-Soc-Clin-Oncol) 13: 300, Mar 1994.
PUBLICATION DATE: 1 March 1994 (**19940301**)
LANGUAGE: English
ADIS LMS: Cancer Chemotherapy (Index only): Alert no. 11, 1994
RECORD TYPE: Citation
DOCUMENT TYPE: Clinical study, abstract

DESCRIPTORS: Biotechnology; Breast-cancer; Monoclonal-antibody-2B1, pharmacodynamics; Research-and-development

5/7/161 (Item 3 from file: 173)
DIALOG(R)File 173:Adis LMS Drug Alerts
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00163770 800164344
TITLE: Antitumor effects of a **bispecific antibody** targeting CA19-9 antigen and **CD16**.
AUTHOR: Garcia de Palazzo I; Holmes M; Gercel Taylor C; et al
JOURNAL: Cancer Research (Cancer-Res) 52: 5713-5719, 15 Oct 1992.
PUBLICATION DATE: 15 October 1992 (**19921015**)
LANGUAGE: English
ADIS LMS: Cancer Chemotherapy (Index only): Alert no. 11, 1992
RECORD TYPE: Citation
DOCUMENT TYPE: In vitro

DESCRIPTORS: Biotechnology; Cancer; Monoclonal-antibodies, pharmacodynamics; Research-and-development

5/7/165 (Item 2 from file: 357)
DIALOG(R)File 357:Derwent Biotechnology Abs
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0169666 DBA Accession No.: 94-12217 PATENT
Production of hybridoma cell lines producing anti-CD16 antibodies - tetradoma construction, bispecific antibody production and use in e.g. Hodgkin disease therapy
PATENT ASSIGNEE: Biotest-Pharma **1994**
PATENT NUMBER: DE 4337197 PATENT DATE: 940825 WPI ACCESSION NO.: 94-256364 (9432)
PRIORITY APPLIC. NO.: DE 4337197 APPLIC. DATE: 931030
NATIONAL APPLIC. NO.: DE 4337197 APPLIC. DATE: 931030
LANGUAGE: German
ABSTRACT: A process is claimed for the selective production of hybridoma cell lines which produce an IgG1 mouse monoclonal antibody (MAb) directed against the human CD16 antigen which has a high capacity for

Set	Items	Description
S1	12837	FC(5N)RECPETOR? OR CD16
S2	2118	S1(15N)ANTIBOD?
S3	298	S2(15N)BISPECIFIC
S4	220	S3/1980:1994
S5	173	RD (unique items)

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1) Snider et al.
J. IMMUNOLOGY
Vol. 139
p. 1609-1616
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2) Palazzo et al.
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Vol. 52
p. 5713-5719
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3) Snider et al.
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4) Weiner et al.
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p. 227-233
1991

6) Weiner et al.
PROCEEDINGS OF THE AMERICAN SOCIETY OF CLINICAL ONCOLOGY
Vol. 13
p. 300
March 1994

7) Ring, D.B.
"Targeting cytotoxicity with a bi-specific antibody directed to c-erb-2 and human Fc-gamma receptor III-human oncoprotein human Fc-gamma receptor-III CD16 bispecific antibody production, purification, tetradoma 2B1 construction"
ANTIBODY ENG
3pp 1991

ENHANCED ANTIGEN IMMUNOGENICITY INDUCED BY BISPECIFIC ANTIBODIES

By DENIS P. SNIDER,* ANDREAS KAUBISCH, and DAVID M. SEGAL

*From the Experimental Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892; and the *Department of Pathology, McMaster University, Hamilton, Ontario L8N-3Z5, Canada*

Th cells recognize and respond to processed antigen bound to class II MHC molecules on the surfaces of APC (1, 2). In vitro, the targeting of antigen to APC surfaces by heterocrosslinked bispecific antibodies (HBAs)[1] greatly increases the efficiency with which APC endocytose, process, and present antigen to T cells (3–5). Since antibody responses against most protein antigens require T cell help in vivo, we have asked here if HBAs could also enhance the ability of an antigen, in this case hen egg lysozyme (HEL), to induce an antibody response in mice. HBAs were prepared by chemically crosslinking an antibody with specificity for HEL to various other antibodies, each specific for a particular APC cell surface component. Normally, the generation of immune responses after immunization with vaccines and other antigens requires relatively large amounts of antigen, multiple injections, and, in experimental animals, adjuvants (6, 7). By contrast, we show that HBAs, when administered once with nanogram amounts of antigen, in the absence of adjuvant, induce high titers of antibody in mice, and prime mice for a secondary IgG antibody response when rechallenged with soluble antigen.

Materials and Methods

Immunizations and Measurements of Antibody Production. Mice of various strains were given primary injections containing various amounts of HEL (Sigma Chemical Co., St. Louis, MO) or HEL mixed with 5–10 μg of HBA in PBS, in two portions of 50 μl in each hind footpad. Controls received PBS alone. Mice were bled 21 d after the primary injection, and the next day given a secondary challenge of 5–10 μg of HEL (5 μg per hind footpad). 11 d later, the mice were bled again, sera were ia[illegible] and n[illegible] HEL IgG antibody was measured using a solid-phase ELISA. In the ELIS[illegible] HEL was bou[illegible]d to plastic microtiter wells and incubated sequentially wit[illegible] dilutions of [illegible]h test serum, b otin anti-mouse IgG (Southern Biotechnology Assoc., Birmingham [illegible]., avidin-alkaline phosphatase (Sigma Chemical Co.), and finally, substrate (Sigma C[illegible] Co.). The amount of antibody in each serum was interpolated from the dose-response cu ve of a standard pool of hyperimmune sera, giving an arbitrary value of antibody U/ml

Antibodies. All HBAs used in this paper were prepared by chemically crosslinking mAbs with succinimidyl-3-(2 pyridyldithiol)propionate as described (8). HBAs were separated from uncrosslinked monomeric antibodies by gel filtration and are designated "antibody 1 × anti-

Address correspondence to David M. Segal, Experimental Immunology Branch, National Cancer Institute, Building 10, Room 4B17, National Institutes of Health, Bethesda, MD 20892.

[1] *Abbreviations used in this paper:* HBA, heterocrosslinked bispecific antibodies; HEL, hen egg lysozyme.

body 2." For example, anti-I-A^b × anti HEL refers to a gel filtration fraction of 300 kD or greater, obtained when anti-I-A^b was crosslinked to anti-HEL. Antibodies used in this paper are anti-HEL, HyHEL-8 (9); anti-I-A^b, 10-2.16 (10); anti-I-A^d, MKD6 (11); anti-K^bD^d, 34.1.2 (12); anti-K^k, 36.7.5 (12); anti-Fc_γRII, 2.4G2 (13); anti-IgD^a, AM8.28.1 (14); and anti-IgD^b, AF3-33.3 (14). Fab fragments were produced by papain digestion and were purified by gel filtration followed by ion exchange chromatography (15).

Results and Discussion

Female A/J mice were given subcutaneous injections of graded amounts of HEL or HEL mixed with HBAs, on day 0. 21 d later, the mice were bled to determine serum anti-HEL IgG antibody levels. On day 22, the mice were given booster injections of 5 μg of HEL per hind foot pad, to induce secondary IgG antibody responses. Sera were collected 11 d later and IgG antibody was measured. Fig. 1 shows that >10 μg of HEL per mouse was required in a primary injection to produce either a primary or a secondary IgG antibody response, if the HEL was given alone. However, in the presence of 10 μg of HBA (anti-I-A^b × anti-HEL), 100 ng of HEL induced a substantial primary IgG antibody response, and as little as 10 ng of HEL induced a strong secondary response. In other experiments (e.g., Fig. 2), <1 ng of HEL in the presence of HBA gave a significant secondary response. Mice receiving PBS as a primary injection, and then given a secondary challenge of HEL (Fig. 1), or given the HBA alone as a primary injection and then a secondary challenge of HEL (Fig. 2), produced little or no IgG antibody. We compared immunization using HBAs with that of HEL emulsified in IFA (Fig. 2). The two types of immunization produced similar amounts of IgG antibody in the secondary response, especially at low HEL doses. Thus, the HBA specific for HEL and class II MHC structures increased the efficiency of immunization by 300-fold in the primary response, and by a factor of 10^3 to 10^4 after a secondary boost. The HBA increased the efficiency of priming for secondary IgG antibody responses at least as well as IFA.

We constructed several other HBAs that could bind to different surface molecules

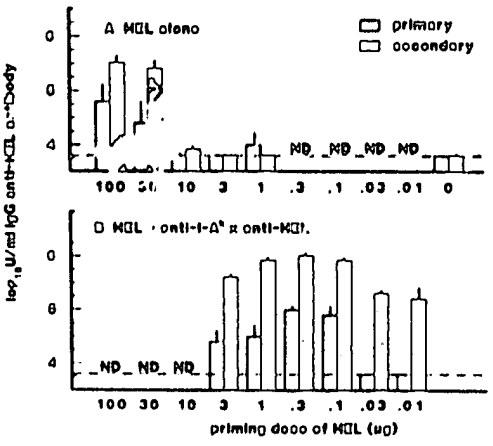

FIGURE 1. HBA enhancement of antibody responses in vivo. Groups of three female, 7-wk-old A/J mice were given single primary foot pad injections of (*A*) HEL alone or (*B*) HEL mixed with 10 μg HBA. The open bars indicate serum IgG anti-HEL antibody levels 21 d after the primary injection. The solid bars indicate antibody levels in sera taken 11 d after these same mice were given a secondary injection of 10 μg HEL without the HBA. The dashed line indicates the lower limit of detection.

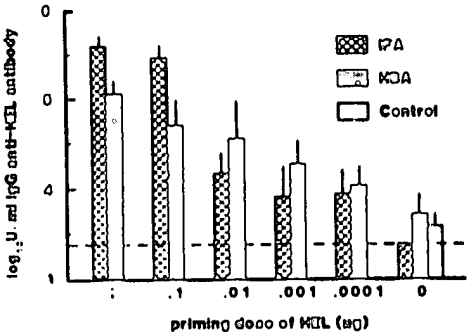

FIGURE 2. Enhancement of antibody production by HBA and IFA. Secondary antibody responses are shown for groups of four female, 6-wk-old B10.A mice given primary injections of HEL mixed with 10 μg HBA (*solid bars*) or emulsified (1:1 [vol/vol]) in IFA (*cross-hatched bars*). Control mice (*open bar*) received HBA but no HEL as a primary injection. The horizontal dashed line indicates the limit of detection of antibody.

on various APC, and used them to immunize mice (Table I). HBAs specific for class I or class II MHC molecules, or $Fc_\gamma RII$, all enhanced the ability of HEL to induce antibody responses in different strains of mice. However, two HBAs with specificity for two allotypes of IgD when tested in several strains of mice induced little or no anti-HEL antibody (Table I, Exp. 2). In other experiments (not shown), we were unable to generate a significant anti-HEL response by varying the dosages of the IgD-specific HBAs and HEL from 20 to 0.1 μg. These same IgD-specific HBAs enhance the efficiency of presentation of HEL to Th hybridomas by B cells in vitro

TABLE I

HBA with Specificity for Class I or II MHC Molecules and for $Fc_\gamma RII$, but not for sIgD, Enhance Humoral Responses Against HEL

Exp.	Mouse strain	Haplotype KID	HBA in primary injection	IgG anti-HEL responses	
				Primary	Secondary
				U/ml × 10^{-3}	
1	CAF1	ddd × kkd	-	7(0)*	7(0)
			Anti-I-A^k × anti-HEL	8(1)	166(60)
			Anti-K^dD^d × anti-HEL	7(0)	33(11)
			Anti-$Fc_\gamma RII$ × anti-HEL	8(1)	40(15)
	A/J	kkd	-	ND	9(4)
			Anti-I-A^k × anti-HEL	63(15)	933(200)
			Anti-K^k anti-HEL	14(12)	302(115)
			Anti-Fc_γ I × anti-HEL	13(11)	269(70)
2	CAF1	kkd	-	2(0)	3(2)
			Anti-I-A^k × anti-HEL	19(6)	118(14)
			Anti-IgD^a × anti-HEL	2(0)	9(4)
	B10.BR	kkk	-	2(0)	3(0)
			Anti-I-A^k × anti-HEL	19(10)	39(3)
			Anti-IgD^b × anti-HEL	3(1)	12(2)

Groups of 4–5 female mice of different strains were given primary injections of 2 μg of HEL with or without 10 μg of HBA, having the indicated specificities. After 21 d, each mouse was given a second injection of 10 μg of HEL in PBS. Sera were collected 11–13 d later, and anti-HEL IgG antibody was measured and is shown as U/ml. The limits of detection were 7,000 U/ml for Exp. 1 and 2,000 U/ml for Exp. 2.

* Figures in parentheses are SEM.

(4). The inability of anti-sIgD-containing HBAs to enhance immunogenicity suggests that either these HBA antigen complexes, when given subcutaneously, do not bind to B cells in vivo (e.g., they might be cleared rapidly or circulating IgD might block their binding to B cells), or that B cells are not able to stimulate unprimed T cells in vivo. B cells appear to serve as in vivo APC to stimulate T cell proliferation in other systems (16–20).

Several important controls for the effect of HBAs are shown in Table II. First, HBAs enhanced antibody production only if the appropriate antibodies were in fact crosslinked; monomeric mixtures of antibodies did not work (Table II, Exp. 1). Second, antibody was induced only if the strain of mouse immunized expressed the structures to which the HBA could bind (Table II, Exp. 2). Thus, HBAs with I-A^d or K^dD^d specificities did not produce responses in H-2^k (C3H) mice, but did produce responses in H-2^k × H-2^d (CAF1) mice. The same I-A^d-specific HBA did not induce antibody in A/J mice, but the K^dD^d-specific HBA did, since this strain expresses D^d but not I-A^d. Both the C3H and the A/J mice responded when immunized with the HEL- and Fc_γRII-specific HBA.

Because antigen targeted to Fc_γRII gave enhanced antibody responses to HEL in several strains, it was important to show that the HBAs were not simply forming immune complexes with HEL and binding via their Fc portions to Fc_γR on APC. Therefore, we constructed an HBA from Fab fragments of anti-HEL and anti-I-A^k mAbs, and compared its ability to induce anti-HEL antibody with that of its intact Ig counterpart (Table II, Exp. 3). The results show that the Fab-HBA performed as well as the intact Ig-HBA over a range of dosages, indicating that the Fc regions of the HBA were not required for enhanced antibody production. This conclusion is also supported by the observation that anti-H-2^d-specific HBAs did not promote antibody production in H-2^k (i.e., C3H) mice (Table II, Exp. 2).

It is likely that HBAs increase anti-HEL production in mice by targeting HEL to surface structures on APC in vivo. Such targeting would cause the antigen to be processed and presented to T cells with much higher efficiency than antigen given alone, as documented in vitro (3–5). Presumably, such APC could then generate large numbers of antigen-specific Th cells, which in turn would stimulate antigen-specific B cells. The known T cell dependence of anti-HEL responses in mice (21, 22) supports this view.

Our use of HBAs to enhance immunization follows on previous results (18, 23–25), showing that antigens that were chemically crosslinked directly to antibodies specific for class II MHC or IgM molecules were more efficient immunogens than antigens given alone. HBAs provide several important advantages over chemically linked antigen-antibody conjugates. First, HBAs could be used to enhance responses agninst small amounts of impure antigens, for example, tumor antigens, whereas direct linkage of antigen to antibody requires relatively large amounts of highly purified antigen. HBAs do not require biochemical modification of antigen, which could alter its immunogenicity. In addition, bispecific antibodies in the form of hybrid-hybridomas (26, 27) or hetero-F(ab')$_2$ (28) can be prepared as single homogeneous species, whereas antibody-antigen chemical complexes are heterogeneous, and vary between preparations. Therefore, homogeneous bispecific antibodies would be much more suitable for large scale production than the antigen-antibody chemical complexes. Finally, we have found that the microgram amounts of HBAs used in this study are

TABLE II

Form, Specificity, and Dose of HBA Required for Enhancement of Humoral Responses

Exp.	Mouse strain	Antibody (form)	Ab dose	HEL dose	Secondary response: IgG anti-HEL
			μg	μg	U/ml × 10^{-3}
1	A/J	Anti-I-A^b × anti-HEL (HBA)	5	1	250(59)*
		Anti-I-A^b + anti-HEL (mix)	5	1	9(3)
		Anti-Fc_γRII × anti-HEL (HBA)	5	1	120(21)
		Anti-Fc_γRII × anti-HEL (mix)	5	1	11(2)
		Anti-HEL (mAb)	2.5	1	10(3)
		None	0	1	11(6)
		None	0	0	7(0)
2	CAF1	Anti-I-A^d × anti-HEL (HBA)	10	3	240(65)
		Anti-K^dD^d × anti-HEL (HBA)	10	3	174(70)
		None	0	3	3(0)
	C3H/HeJ	Anti-I-A^d × anti-HEL (HBA)	10	3	8(4)
		Anti-K^dD^d × anti-HEL (HBA)	10	3	5(3)
		Anti-Fc_γRII × anti-HEL (HBA)	10	3	245(95)
		None	0	3	11(5)
	A/J	Anti-I-A^d × anti-HEL (HBA)	10	3	18(9)
		Anti-K^dD^d × anti-HEL (HBA)	10	3	112(69)
		Anti-FcR_γRII × anti-HEL (HBA)	10	3	302(122)
		None	-	3	13(2)
3	A/J	Anti-I-A^b × anti-HEL (HBA)	1	2	417(129)
		Anti-I-A^b × anti-HEL (HBA)	3	2	525(183)
		Anti-I-A^b × anti-HEL (HBA)	10	2	372(55)
		Anti-I-A^b × anti-HEL (Fab-HBA)	1	2	1,122(440)
		Anti-I-A^b × anti-HEL (Fab-HBA)	3	2	603(209)
		Anti-I-A^b × anti-HEL (Fab-HBA)	10	2	587(124)
		None	0	2	1(0)

Groups of female mice (4–5 per group) were injected with the indicated doses of HEL or antibody. In Exp. 1, the effect of HBAs on the anti-HEL antibody response was compared with that of the uncross-linked fraction (mix) obtained during purification of HBAs, and to that of the anti-HEL mAb alone. In Exp. 2, the specificity of targeting was tested in mouse strains that express structures to which particular HBAs bind or do not bind. C3H/HeJ and A/J mice were also immunized with HEL plus an anti-Fc_γRII-specific HBA as a control for their ability to respond to targeted HEL. The isotypes and allotypes of antibodies used in this experiment were as follows: anti-I-A^d (MKD6), mouse IgG2a, IgH-1-b or -e (strain of origin, B6 × A/J); anti-K^dD^d (34.1.2), mouse IgG2a, IgH1-j (strain of origin, C3H); anti-HEL (HyHEL-8), mouse IgG1, IgH4-a (strain of origin, BALB/C); anti-Fc_γRII (2.4G2), rat IgG2b. There is no obvious relationship between possible alloreactivities against HBAs and their ability to enhance anti-HEL production. In Exp. 3, anti-I-A^b anti-HEL HBAs prepared from intact antibodies or from Fab fragments were compared for their ability to induce an HEL antibody. In all experiments, mice were bled 11–14 d after a secondary injection of 10 μg of HEL in PBS. Data are mean U/ml of serum IgG antibody. The limits of detection were 7,000 U/ml (Exp. 1), 3,000 U/ml (Exp. 2), and 1,000 U/ml (Exp. 3).

* Figures in parentheses are SEM

remarkably potent enhancers of immunogenicity, leading to priming with nanogram amounts of antigen. Such enhancements of immunogenicity are far greater than those reported in studies using antigen-antibody chemical complexes. Our observations that relatively small amounts of HBAs can enhance antibody responses at least as well as IFA suggests that HBAs may be useful in immunizing man and domestic animals against bacterial, viral, parasitic, and, perhaps, tumor antigens. Such im-

munization would be efficient and would avoid the use of adjuvants that have unwanted inflammatory effects (29), and cannot, therefore, be used in humans.

Summary

The binding of protein antigens to APC with heterocrosslinked bispecific antibodies (HBAs) enhances their processing and presentation to Th cells in vitro. Here we have asked whether HBAs could also increase immune responses in vivo. We immunized mice with hen egg lysozyme (HEL) in the presence or absence of HBA, and followed antibody production after the primary challenge and after a secondary boost. We found that HBAs that bind antigen to MHC class I or II molecules, to $Fc\gamma R$, but not to surface IgD, enhance the immunogenicity of HEL. HBAs that bound HEL to MHC class II molecules, for examples, decreased the amount of antigen required to elicit a primary anti-HEL antibody response in mice by 300-fold, and the amount required to prime for a secondary response by 10^3- to 10^4-fold. In fact, HBAs were as effective as IFA in generating antibody responses. Since adjuvants cannot be used in humans, HBAs could prove useful for immunizing people, especially in cases where, due to scarcity or toxicity, minute doses of antigen must be used.

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1305

A Phase I clinical trial in cancer patients with a new monoclonal antibody (MAb), FC-2.15. Mordoh J., Silva C., Albarellos M. & Kairiyama C. Instituto de Investigaciones Bioquimicas "Fundacion Campomar" and Instituto Alexander Fleming, Buenos Aires. Argentina.

FC-2.15 is a new murine IgM MAb that recognizes previously undescribed antigens present in proliferating breast cancer cells and normal peripheral granulocytes. A Phase I clinical trial was performed in 10 patients with advanced cancer (breast=4, colon=1, melanoma=2, lung= 1, medullary thyroid=1, skin epidermoid carcinoma=1). FC-2.15 was administered by i.v. infusion every other day ; nine patients received 4 infusions and one patient 3 infusions. One patient received 2 cycles of treatment. Total doses of FC-2.15 ranged between 2.4 and 5.1 mg/kg. Maximal FC-2.15 serum concentration ranged between 2.2 µg/ml and 5.5 µg/ml, and its serum half-life (t1/2) was about 9 hr. All patients developed HAMA. The most consistent side effect (10/10 patients) was a profound and selective neutropenia wich occured within 1 hr after the start of each infusion and reversed within 1 hr after its discontinuation. Other frequent side effects included fever < 38 °C and chills that were easily manageable. None of these effects required dose reduction or treatment interruption ; the patient which received two treatment cycles did not develop allergic reactions. A complete response of a hepatocellular carcinoma and a carcinoid (6 months) >50 % partial response of breast carcinoma liver metastases were observed.

1306

Radioimmunodetection of epithelial cancers with an anti-MUC1 murine monoclonal antibody. Sharkey, R.M., Dion, A.S., Swayne, L.C., Conte, P., Markowitz, A., and Goldenberg, D.M. Garden State Cancer Center at the Center for Molecular Medicine and Immunology, and St. Michael's Newark, NJ 07103, and Morristown Memorial Hospital, Morristown, NJ 07950.

We have investigated an IgG_1 murine monoclonal antibody (MAb) that is reactive with a tandemly repeated peptide epitope of MUC1. Immunohistology indicated that the MAb reacted with 84% (21/25) and 93% (13/14) of primary and metastatic breast cancers, respectively. Also, significantly elevated serum levels of MUC1 were found for at least 50% of breast (all stages), colon, lung, prostate, ovarian, and pancreatic cancer patients. Clinical trials have been initiated to determine the targeting ability of this antibody. ^{131}I-labeled IgG or $F(ab')_2$, as well as ^{99m}Tc-Fab' fragments, have been studied in 5 breast, 3 ovarian and 1 lung cancer patient. Tumors were localized in all but 1 of the breast cancer patients. In 2 patients, disease was first seen by radioantibody imaging with subsequent confirmation by magnetic resonance imaging. Although MUC1 is found in the plasma, < 25% of radiolabeled antibody in the plasma was complexed after injection in only 3/6 assessible patients. These studies suggest that this anti-MUC1 MAb may be a useful antibody for detection or therapy of epithelial cancers. (Supported in part by PHS grant CA39841.)

1307

Initial clinical investigations of PAM4, a new murine monoclonal antibody against pancreatic cancer. Sharkey, R.M., Gold, D.V., Markowitz, A., Swayne, L.C., Conte, P., and Goldenberg, D.M. Garden State Cancer Center at the Center for Molecular Medicine and Immunology and St. Michael's Hospital, Newark, NJ 07103, and Morristown Memorial Hospital, Morristown, NJ 07950.

A new murine monoclonal antibody designated PAM4 was generated against a mucin from pancreatic cancer. By immunohistology, PAM4 showed reactivity with greater than 80% of pancreatic cancers and yet was unreactive with normal adult pancreatic tissue. Animal studies showed excellent targeting with ^{131}I-labeled PAM4 (21 to 44 percent of injected dose per gram of tumor at day 3 post injection for 4 different human pancreatic tumor lines). Five patients with proven pancreatic cancer have been studied using ^{131}I-PAM4 IgG or ^{99m}Tc-PAM4 Fab'. Definitive tumor localization was seen in 4/5 patients. Dosimetry from 2 patients receiving ^{131}I-PAM4 IgG predicted tumors received 10-20 cGy/mCi with tumor/red marrow dose ratios ranging from 3-10. These results suggest that PAM4 is a promising antibody for targeting pancreatic cancer. (Supported in part by PHS grants CA39841 and CA54425 and a grant from the Robert Leet and Clara Guthrie Patterson Trust.)

1308

Clinical evaluation of Tc-99m labelled anti-TF MAb 170 in the assessment of metastatic colorectal cancer. Kirsh, J., Smith A., Reilly R., Gallinger S., & Stern H. Departments of Surgery and Nuclear Medicine, Mount Sinai Hospital and The Toronto Hospital, University of Toronto, Toronto, Ontario M5G 1X5.

MAb is an antibody directed against a membrane protein associated with the Thomsen-Friedenreich antigen, expressed on the majority of human adenocarcinomas. Our objective was to evaluate the efficacy of Tc-99m labelled MAb 170 for assessment of metastatic colorectal cancer (MCRC). Twenty patients (6 with concomitant primary CRC) were injected i.v. with 1800 MBq (2 mg) of Tc-99m MAb 170. At 22 h p.i. planar and SPECT images of the abdomen and pelvis were obtained. Sixteen patients (pts) underwent laparotomy within 48 h p.i. and samples of tumor and normal tissues were obtained. Radioactivity in these tissues (%ID/g) was measured by scintillation counting. MAb 170 correctly identified the primary tumor in 3/6 pts and MCRC in 3/5 pts with biopsy confirmation. Two FN studies were in 1 pt with liver metastases and 1 pt with a pelvic nodule. Two additional pts with positive MAb 170 scans await surgical follow-up. CT or US identified MCRC in 4/5 pts. Two pts with negative MAb 170 scans who underwent laparotomy after radiological tests suggested MCRC were found to have non-malignant disease. Two pts with high, rising CEA exhibited positive MAb 170 scans but MCRC could not be identified at laparotomy (FP). Radioactivity in the tumor or normal bowel was 0.00004-0.005%ID/g and 0.00001-0.005%ID/g respectively. We conclude that Tc-99m MAb 170 may have an important role in the assessment of occult MCRC.

1309

Phase I trial of a bispecific murine monoclonal antibody targeting c-erbB-2 and CD16. Weiner, L.M., Ring, D.[1], Li, W., Palazzo, I.E., Davey, M., Rivera, V., Alpaugh, R.K. Fox Chase Cancer Center, Philadelphia, PA 19111, [1]Chiron Corp., Emeryville, CA 94608.

Bispecific monoclonal antibodies (BsMAb) can direct tumor lysis by effector cells via defined cytotoxic trigger molecules. The BsMAb 2B1 promotes c-erbB-2 tumor cell lysis by human NK cells and macrophages expressing CD16 (i.e., FcγRIII), and is effective in murine xenograft models. Neutrophils (PMN) express an isoform of CD16 that does not trigger lysis. In a dose-escalating Phase I clinical trial, nine patients with c-erbB-2(+) tumors have been treated i.v. with 1 hr BsMAb infusions on days 1,4,5,6,7 and 8, at 1 mg/m^2 (n=3) or 2.5 mg/m^2 (n=6) 2B1 per dose. The MTD has not been reached. Treatment causes fevers, rigors, reversible neutropenia and loss of circulating monocytes and NK cells. Treatment also alters the distribution of ^{111}In-labeled autologous leukocytes, with tumor localization noted in at least one patient. Circulating 2B1 retains its dual binding characteristics. Peak levels of 240-2260 ng/ml murine IgG have been detected, with binding to circulating and peritoneal PMN, NK cells and mononuclear phagocytes. One clinical response has been observed in a patient with chest wall recurrence of breast cancer. The binding of this BsMAb to CD16-expressing leukocytes has potent biological effects which may be exploited at higher, tumor-binding 2B1 doses.

1310

Induction of complete remission in advanced refractory chronic lymphocytic leukemia (B-CLL) by CAMPATH-1H with disappearance of malignant trisomy-12 clone determined by fluorescence in situ hybridization (FISH). H. Ozer, A. Steagall, C. Coppedge, M.A. Collier and J. Purvis. University of North Carolina, Chapel Hill, NC 27599 and Burroughs Wellcome Co., RTP, NC 27709.

CAMPATH-1H is under investigation in the therapy of non-Hodgkin's lymphoma and B-CLL. CAMPATH 1-H, a "humanized" version of CAMPATH-1G (secreting IgG2b) is produced by introducing hypervariable regions from the heavy and light chain variable domains of the rodent antibody (Ab) into a human IgG-1 framework. The Ab is directed against CDw52 (CAMPATH-1) antigen which is present on at least 95% of all human peripheral blood B and T lymphocytes and can selectively lyse lymphocytes in the presence of complement. Patient 67 was a 47 year old white male diagnosed in 1984, pretreated for 8 years with pentostatin/interferon, chlorambucil, splenic irradiation, and fludarabine, refractory to each regimen. In 9/92 he developed splenomegaly ≥ 14 cm, WBC > 600,000 and a 4 cm right upper lung mass. He received CAMPATH-1H beginning at 25 mg/m^2 escalated to 75 mg/m^2 tiw IV for 3 months per protocol BW-01. He achieved a complete remission (CR) at 4 weeks and has remained in unmaintained CR for 12 months. Karyotyping in 9/92 confirmed presence of a trisomy 12-positive clone with a normal 46 XY karyotype in 2/93. Absence of minimal residual disease by FISH was documented in 2/93. These data confirm the potential for major clinical and cytogenetic responses in advanced refractory CLL to CAMPATH-1H.

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J. IMMUNOLOGY
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March 1994

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"Targeting cytotoxicity with a bi-specific antibody directed to c-erb-2 and human Fc-gamma receptor III-human oncoprotein human Fc-gamma receptor-III CD16 bispecific antibody production, purification, tetradoma 2B1 construction"
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BISPECIFIC MONOCLONAL ANTIBODIES DIRECTED TO CD16 AND TO A TUMOR-ASSOCIATED ANTIGEN INDUCE TARGET-CELL LYSIS BY RESTING NK CELLS AND BY A SUBSET OF NK CLONES

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CD16 surface antigens represent activatory molecules in $CD3^-16^+$ NK cells. In order to target NK cells against relatively NK-resistant ovarian carcinomas, we used an anti-CD16 monoclonal antibody (MAb) (VD4), together with an anti-ovarian carcinoma-associated antigen (MOV19), to construct biMAbs. To this end, hybrid hybridomas were generated by fusing a TK-deficient VD4 hybridoma mutant with a HGPRT-deficient MOV19 hybrid. Supernatants from hybrid hybridomas that had been selected in HAT medium were screened for their ability to induce a $CD3^-16^+$ NK clone to lyse an $MOV19^+$ ovarian carcinoma cell line in a 4-hr ^{51}Cr-release assay. The NMB.45 hybrid hybridoma secreted a biMAb which triggered lysis of $MOV19^+$ but not of $MOV19^-$ target cells. Some degree of target cell lysis was also observed with MOV19 MAb (due to ADCC mechanisms), while the VD4 MAb was ineffective. HPLC fractionation of MAbs secreted by the hybrid hybridoma made it possible to identify 4 different peaks, one of which appeared to contain functional biMAb molecules. HPLC-purified biMAb (100 ng/ml) induced resting PBL to lyse the "NK-resistant" IGROV1 ovarian carcinoma cell line. Fresh $MOV19^+$ tumor cells were also lysed, although with lower efficiency. When IL-2-activated lymphocytes were used as a source of effectors, biMAb caused only minor increases in the IL-2-induced cytolytic activity. Further analyses of the effect of biMAb were performed at the clonal level. Among $CD3^-16^+$ NK cell clones, a clear enhancing effect could be observed only in $GL183^+$ but not in $GL183^-$ clones. In $CD3^+$ cytotoxic clones a triggering effect could be detected in one out of 4 TCR γ/δ^+ clones but not in TCR α/β^+ clones.

Natural killer (NK) cells represent a subset of lymphocytes which constitutively express MHC-unrestricted cytolytic activity against a number of "NK-susceptible" tumor target cells (Herberman and Ortaldo, 1981; Trinchieri and Perussia, 1984; Trinchieri, 1989). However, the majority of fresh or cultured tumor cells are relatively "resistant" to the activity of fresh peripheral blood NK cells and they become susceptible to lysis following NK-cell activation by culture in IL-2 (Phillips and Lanier, 1986; Ferrini *et al.*, 1987). NK cells are characterized by the absence of a CD3/T-cell receptor (TCR) complex and by the expression of CD56 and CD16 (Fcγ-receptor III) surface markers (Hercend *et al.*, 1985; Lanier *et al.*, 1986). The CD16 molecule is physically linked to CD3ζ chain at the NK cell surface to form a signal-transducing receptor complex (Anderson *et al.*, 1989; Lanier *et al.*, 1989). Binding of appropriate ligands (such as anti-CD16 antibodies or complexed IgG) to CD16 molecule results in NK-cell activation and triggering of cytolytic functions (Lanier *et al.*, 1988; Moretta *et al.*, 1989) or lymphokine secretion (Anegon *et al.*, 1988; Cassatella *et al.*, 1989).

MAbs directed against a number of triggering surface molecules of T lymphocytes have been used in conjunction with MAbs directed to tumor-associated antigens to construct bispecific monoclonal antibodies (biMAbs) (Perez *et al.*, 1985; Staerz and Bevan, 1986; Lanzavecchia and Scheidegger, 1987; Mezzanzanica *et al.*, 1988; Ferrini *et al.*, 1989*a*). In view of their double specificity, biMAbs are able to target effector lymphocytes against appropriate tumor target cells. It should be noted that induction of cytolysis by biMAbs requires that effector cells should possess a functional cytolytic machinery. Therefore, efficient targeting of T lymphocytes by biMAbs is dependent upon previous *in vitro* activation (Pupa *et al.*, 1988; Garrido *et al.*, 1990).

Since NK cells constitutively express cytolytic functions, we have analyzed whether anti-CD16/anti-tumor biMAbs, produced by a hybrid/hybridoma, could target NK cells against relevant tumor cells. We show that resting NK cells can be targeted by biMAbs against "NK-resistant" ovarian carcinoma cells. In addition, analysis of a large number of NK clones revealed that anti-CD16 biMAbs efficiently triggered tumor-cell lysis by NK clones expressing the subset-specific GL183 surface marker.

MATERIAL AND METHODS

Selection of T- and NK-cell clones

$CD3^-16^+$, $CD3^+$ TCR α/β^+ and $CD3^+$TCR γ/δ^+ clones were derived from peripheral blood lymphocytes (PBL), as previously described (Ferrini *et al.*, 1987). Briefly, cells derived from purified T- and NK-cell populations were cultured under limiting dilution conditions in the presence of autologous irradiated PBL, 1% PHA (vol/vol) and 50 U/ml of human recombinant IL-2 (Cetus, Emeryville, CA) in 96-well microtiter plates. Clones were then expanded in IL-2-containing medium in the absence of feeder cells. Culture medium was RPMI 1640 (Flow, Irvine, UK) supplemented with 10% fetal calf serum and 2 mM L-glutamine.

T- and NK-cell clones were studied for their reactivity with MAbs by indirect immunofluorescence and FACS analysis. The MAbs used in these experiments were: Leu4 (anti-CD3), Leu2a (anti-CD8), Leu-11c (anti-CD16) (Becton Dickinson, Milan) WT31 specific for a common determinant of the TCR α/β (Sanbio, Uden, The Netherlands), BB3 specific for a major subset of TCR γ/δ^+ lymphocytes (Ciccone *et al.*, 1988) and GL183, specific for a subset of NK cells (Moretta *et al.*, 1990).

An FITC-conjugated goat anti-mouse IgG antiserum was used as second reagent.

Enzyme-deficient hybridoma mutants

The VD4 (IgG_1) and MOV19 (IgG_{2a}) hybridomas were used for these studies. VD4 produces an anti-CD16 MAb, which triggers cytolytic activity by NK cells (Moretta *et al.*, 1989). The Mov19 hybridoma is specific for a 38-kDa surface antigen selectively expressed on human ovarian carcinoma cells (Mi-

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otti *et al.*, 1987). Hypoxanthine-guanine phosphoribosyltransferase (HGPRT)-deficient mutants of the MOV19 hybridoma were selected by culture in the presence of increasing amounts of 8-azaguanine (1 to 50 μg/ml) (Sigma, St. Louis, MO). Thymidine kinase (TK)-deficient mutants of VD4 hybridoma were obtained by selection in the presence of 5-bromodeoxyuridine (1 to 60 μg/ml) (Sigma). Hybrid mutants were cloned by limiting dilution and tested for HAT sensitivity and antibody production. Culture medium for hybrids was D-MEM supplemented with 10% FCS and 2 mM L-glutamine.

Cell fusion and screening of hybrid hybridomas

A HGPRT-Mov19 clone was fused with a TK^- VD4 hybridoma at a 1:1 ratio using a standard polyethylene glycol fusion protocol. Hybrid hybridomas were selected by culture in HAT medium. Both TK- and HGPRT-deficient cells die under these selection conditions, while hybrid hybridomas complement each other and survive. Culture supernatants were tested for their ability to trigger a $CD3^-16^+$ NK clone to lyse the $Mov19^+$ IGROV1 target-cell line in a 4-hr assay.

Assay for cytolytic activity

PBL, T and NK clones were used as effector cells at effector:target cell ratios ranging from 40:1 to 0.6:1 in a 4-hr ^{51}Cr-release assay. The following tumor cells were used as targets: IGROV1 (ovarian carcinoma) (Bénard *et al.*, 1985), MeWo (melanoma), Raji (Burkitt lymphoma), and fresh ovarian carcinoma cells isolated from ascites fluid of stage II–IV ovarian carcinoma patients as previously described (Ferrini *et al.*, 1985). Briefly, 5×10^3 ^{51}Cr-labelled target cells were added to various numbers of effector cells in a 96-well microtiter plate. For the evaluation of MAb-triggered cytotoxicity, various dilutions of hybrid supernatant or of HPLC-fractionated MAb were added at the onset of the assay. The final culture volume was 200 μl. After 4 hr of culture, 100 μl of supernatant were collected from each well and counted in a gamma counter for the evaluation of ^{51}Cr-release. The percentage of lysis was calculated (Ferrini *et al.*, 1987).

High-performance liquid chromatography (HPLC separation)

HPLC separation of the (protein-A Sepharose) purified biMAb was carried out on a 7.6 × 100 mm hydroxyapatite prepacked column (HCA-column) (Mitsui Toatsu, Tokyo, Japan).

Samples, filtered through a 0.45-mm HV filter (NIHON, Millipore, Kogyo, Yonezawa, Japan), were eluted by 50-min concave gradient from 0.01 M (buffer A) to 0.35 M (buffer B) potassium phosphate buffer, pH 6.8. Proteins (0.035 and 0.5 mg) were run in analytical and preparative separations, respectively, at a flow rate of 1 ml/min. Fractions of 1 ml were collected and E_{254nm} analyzed using a liquid chromatography spectrophotometer (Waters, Milford, MA).

Two-color immunofluorescence analysis

The IGROV1 cell line and a $CD16^+$ clone were stained with one of the following MAbs: MOV19, VD4, NMB.45, and with peaks isolated from HPLC fractionation of the NMB.45 biMAb. A phycoerythrin (PE)-conjugated anti-IgG_1 and a fluorescein-conjugated anti-IgG_{2a} goat antiserum (Southern Biotechnologies, Birmingham, AL) were used as second reagents. Cytofluorimetric analysis was performed on a FACS-Star (Becton Dickinson).

RESULTS

Selection of hybrid hybridomas producing anti-CD16/anti-tumor biMAbs

In order to construct hybrid hybridomas producing biMAbs, the TK-deficient VD4 anti-CD16 hybridoma was fused with the HGPRT-deficient MOV19 hybridoma. The MOV19 MAb is directed against a tumor-associated antigen specifically expressed by ovarian carcinoma cells. Hybrid hybridomas were selected in HAT medium and the resulting supernatants were directly screened for their ability to induce the CK8 $CD3^-16^+$ NK cell clone to lyse the $MOV19^+$ IGROV1 ovarian carcinoma cell line. The CK8 clone was selected from a panel of NK clones because of its low level of cytolytic activity against the IGROV1 cell line (in the absence of added MAb). The hybrid hybridoma NMB.45 was selected according to this screening criterion and was repeatedly subcloned by limiting dilution. As shown in Figure 1*a*, a 1/100 dilution of the hybrid hybridoma supernatant efficiently induced the CK8 clone to lyse the relevant tumor target. Although the MOV19 parental MAb alone induced some degree of target-cell lysis (due to ADCC mechanisms), noticeably higher levels of lysis were induced by NMB.45 biMAb. On the other hand, anti-CD16 MAbs alone were ineffective (lack of appropriate Fc receptors on the tumor target cells utilized).

When the Mov19-negative (Fc-receptor-negative) MeWo and Raji cells were utilized as targets, no cytolytic activity was induced by the NMB.45 biMAb (Fig. 1*b*), thus indicating that $CD16^+$ effector cells could only be targeted on tumor cells expressing the relevant antigen.

Purification of biMAbs by HPLC

Due to the random association of Ig chains synthesized by hybrid hybridomas, it is evident that the relevant biMAb will represent only a fraction of the antibody molecules secreted by the hybrid hybridoma (Milstein and Cuello, 1983, 1984). In an attempt to purify biMAb from the other antibody molecules produced by the NMB.45 hybrid hybridoma, HPLC fractionation of secreted MAbs was performed on a hydroxyapatite column. As shown in Figure 2, 4 different peaks could be identified. The major peak (peak 4) corresponded to the elution pattern of the parental MOV19 MAb, while none of the peaks had an elution time corresponding to that of the parental VD4 MAb. In order to detect the presence of functional biMAbs in the different fractions, 100 ng/ml of protein from each peak were analyzed for their ability to induce lysis of IGROV1 target cells by the CK8 clone. As shown in Table I, peaks 1 and 2 failed to trigger cytolysis, peak 4 had only a partial effect, while peak 3 induced maximal cytolytic activity.

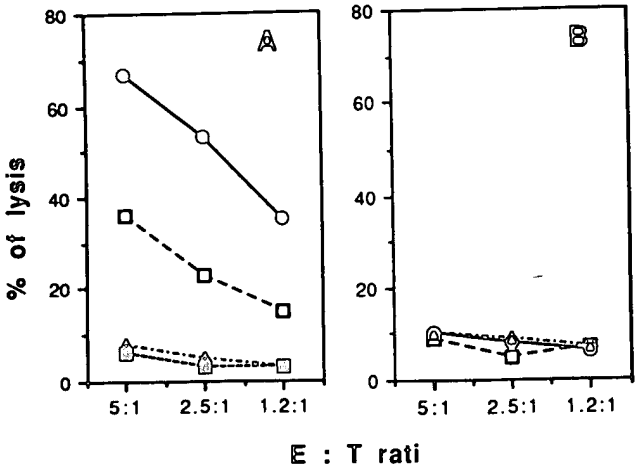

FIGURE 1 – Effect of the biMAb on the cytolytic activity of the $CD3^-16^+$ clone CK8 against the $MOV19^+$ IGROV1 *(a)* and the $MOV19^-$ MeWo *(b)* target cells in a 4-hr ^{51}Cr release assay. Cytolytic activity in the presence of: —○— hybrid hybridoma supernatant (1:100 final concentration); – – □ – –, Mov19 MAb; - - -□- - -, VD4 MAb; – · –△– · —, no antibody.

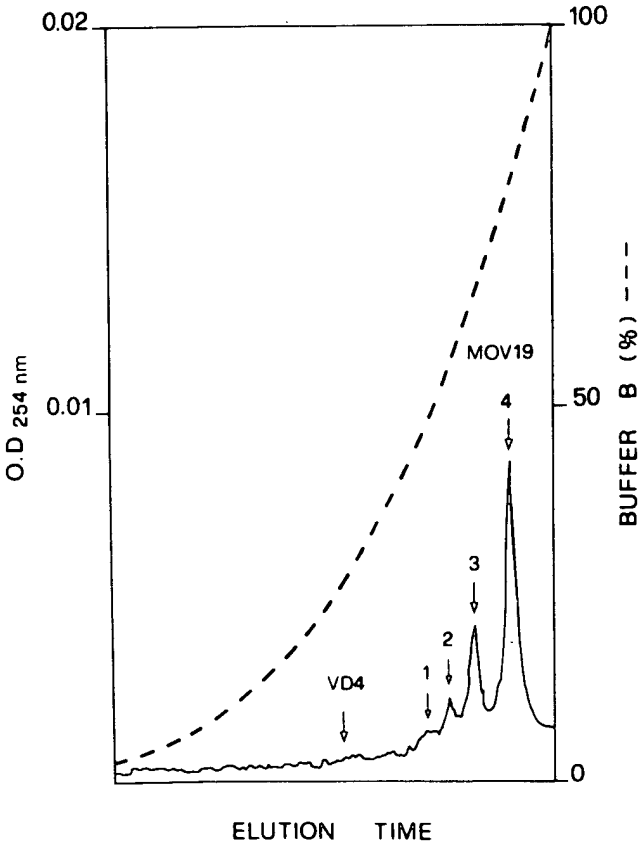

FIGURE 2 – HPLC elution profile of antibody molecules secreted by the NMB.45 hybrid hybridoma. Protein A–Sepharose purified antibody was loaded on a hydroxyapatite column and eluted with a gradient from 0.01 M to 0.35 M potassium phosphate buffer (pH 6.8).

TABLE I – EFFECT OF THE NMB.45 SUPERNATANT OR OF HPLC-PURIFIED MAb FRACTIONS ON THE CYTOLYTIC ACTIVITY OF A $CD3^-16^+$ CLONE

E/T ratio	Control	MOV19	NMB.45 supernatant	Peak:1	2	3	4
5:1	6	33	67	6	5	70	43
2.5:1	2	21	53	3	4	53	28
1.2:1	1	11	36	1	6	36	16

Data are expressed as percentage ^{51}Cr release from IGROV1 target cells. The $CD3^-16^+$ clone CK8 was used as effector.

Since the VD4 and MOV19 parental MAbs belonged to 2 different Ig isotypes (IgG_1 and IgG_{2a}, respectively) the presence of hybrid IgG_1/IgG_{2a} antibody molecules in the different HPLC fractions could be studied by the use of 2-color immunofluorescence using an anti-IgG_1 PE- and an anti-IgG_{2a} FITC-conjugated antiserum. As shown in Figure 3, no fluorescence could be detected when IGROV1 cells were stained with peak 1 and 2 (panels C and D) and only green fluorescence was observed with peak 4 (panel F), while most IGROV1 cells were double-stained by peak 3 (panel E). Staining of a $CD16^+$ clone with the various peaks revealed double fluorescence only with peak 3 (data not shown). Taken together, these results clearly indicate that functional IgG_1/IgG_{2a} molecules, reacting with both IGROV1 and NK cells, were formed in the NMB.45 hybrid hybridoma and that these molecules eluted in a single peak after HPLC fractionation. It is conceivable that peaks 1 and 2 were represented by MAbs resulting from non-functional reassociations of the VD4 and MOV19 heavy and light chains.

We also analyzed the ability of different concentrations of purified biMAb (peak 3) to induce cytolytic activity. Dose-response curve analysis (Fig. 4) showed that as little as 250 ng/ml of purified biMAb were maximally effective and that triggering of cytolysis was still evident at the lowest concentration tested.

Since the induction of cytolysis by the NMB.45 biMAb could be related to an increased affinity of the hybrid IgG_1/IgG_{2a} Fc portion for the human Fc-receptor III (CD16), further experiments were performed to exclude this possibility. To this end we utilized another IgG_1/IgG_{2a} biMAb, termed AM18.4 (Ferrini *et al.*, 1989*a*), constructed (by the hybrid hybridoma technique), with the same anti-tumor MAb (MOV19) and with an MAb directed against a Vδ1-associated determinant expressed by a subset of TCR γ/δ^+ lymphocytes (Ferrini *et al.*, 1988*b*; Miossec *et al.*, 1990). As shown in Table II, the HPLC-purified AM18.4 biMAb failed to induce ADCC, by resting NK cells or by a $CD3^-16^+$ NK clone, although it efficiently triggered cytolysis by a $V\delta1^+$ TCR γ/δ^+ clone. These data indicate that hybrid IgG_1/IgG_{2a} murine MAb molecules display a reduced affinity for human Fc-receptor III, as compared to IgG_{2a}, and thus support the notion that induction of target-cell lysis by NMB.45 biMAb is not mediated by an ADCC mechanism.

Effect of purified biMAb on resting and IL-2-activated PBL

Given the ability of NMB.45 biMAb to trigger the $CD3^-16^+$ NK clone CK8, we next investigated the effect of biMAb on resting NK cells present in fresh PBL. As shown in Figure 5, PBL derived from 3 different donors had virtually no spontaneous cytolytic activity against IGROV1. In the presence of NMB.45 biMAb, the cytolytic activity was clearly enhanced. The effect of biMAb on cytolytic activity was more efficient than ADCC induced in the presence of the parental MOV19 MAb. Fresh $MOV19^+$ ovarian carcinoma cells (isolated from ascites fluids) were more resistant to biMAb-targeted cytotoxicity than cells from the IGROV1 line; however, significant levels of cytotoxicity against fresh tumor cells could be achieved at the highest E/T ratio (Fig. 6).

Following culture in IL-2 for 3 days, PBL acquired non-MHC-restricted cytolytic activity (*i.e.*, lymphokine-activated killer-cell activity) against IGROV1. Addition of biMAb only marginally enhanced cytotoxicity (data not shown).

We further investigated whether the lower level of cytotoxicity observed using fresh tumor target cells was related to a lower expression of the tumor-associated antigen identified by MOV19. FACS analysis performed on IGROV1 and on fresh tumor cells stained with MOV19 MAb showed that the amount of MOV19 antigen expressed by the fresh tumor cell populations utilized in this study was similar to that of IGROV1 cells (data not shown). Fresh ovarian carcinoma cells were more resistant than ovarian carcinoma cell lines (IGROV1 and SW626) to cytolysis mediated by IL-2-activated PBL (data not shown) and by TCR γ/δ^+ cells targeted with anti-TCR γ/δ biMAbs (Ferrini *et al.*, 1989*a*).

Effect of biMAb on T- and NK-cell clones

Although the NMB.45 biMAb had a marginal enhancing effect on the cytolytic activity of IL-2-activated PBL, the clear-cut induction of lysis observed on the CK8 clone suggested that heterogeneity could exist among different types of IL-2-activated effector cells in their susceptibility to triggering signals delivered by biMAb. In order to investigate this possibility, we analyzed a panel of different T- and NK-cell clones (Table III). Among $CD3^-16^+$ clones we distinguished 2 different subsets, as defined by the expression of the recently described GL183 surface molecule (Moretta *et al.*, 1990). As reported (Moretta *et al.*, 1990) $GL183^+$ and $GL183^-$ NK-cell clones differed in their ability to lyse tumor cells. Thus, while

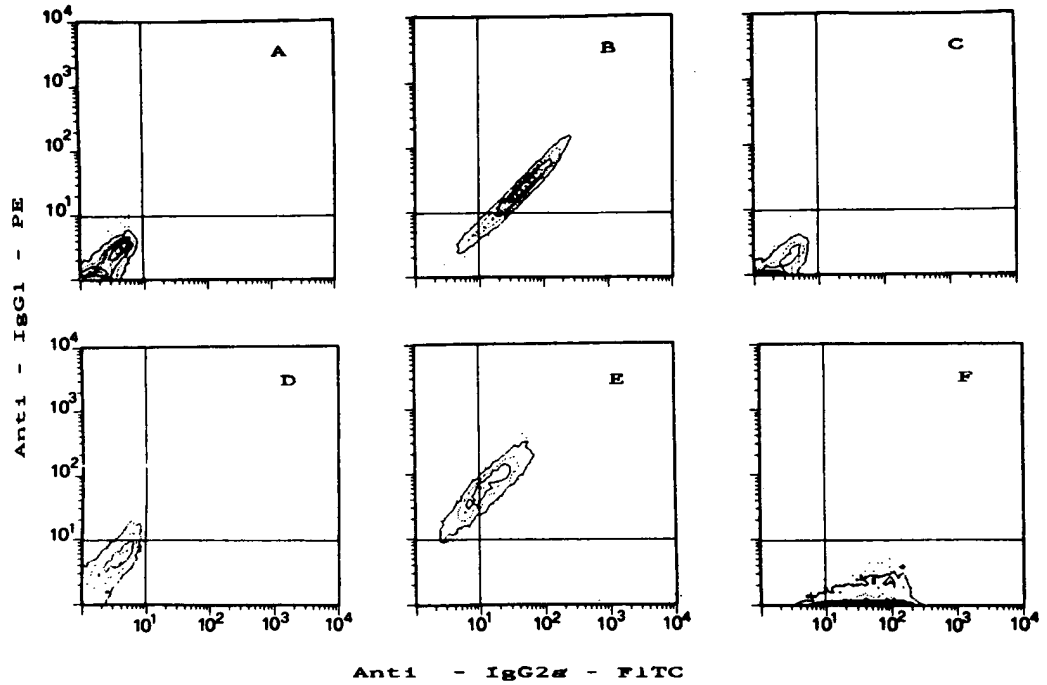

FIGURE 3 – Two-color immunofluorescence analysis of the reactivity of hybrid hybridoma supernatant, or of HPLC-purified MAb fractions, against MOV19$^+$ IGROV1 cells. Cells were incubated without antibody *(a)*, with hybrid hybridoma supernatant *(b)* or with the HPLC peaks 1 to 4 *(c, d, e, f)*. Cells were subsequently stained with a mixture of anti-IgG_{2a} FITC- and anti-IgG_1 PE-conjugated antisera and analyzed on a flow cytometer. In each panel the upper left quadrant contains PE-stained cells, the lower right FITC-labelled ones, the lower left double-negative cells and the upper right double-positive cells.

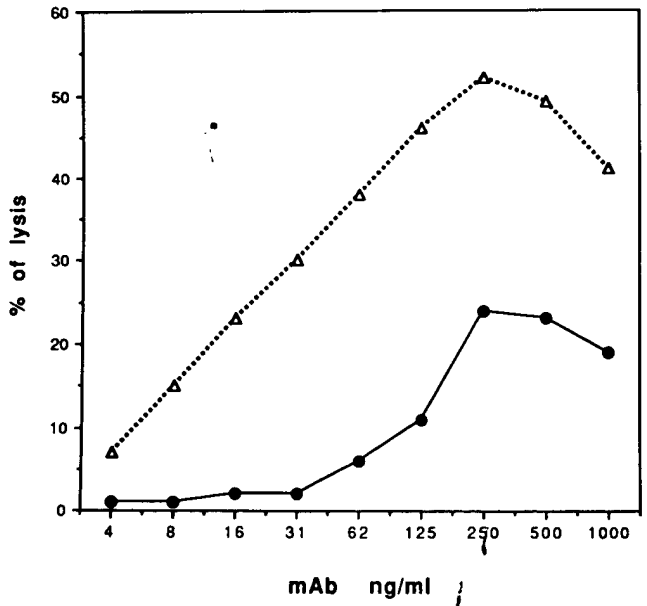

FIGURE 4 – Effect of different concentrations of purified biMAb and of MOV19 parental MAb on the cytolytic activity of a CD3$^-$16$^+$ clone against IGROV1 target cells. Data are expressed as percentage of ^{51}Cr release at an effector/target cell ratio of 2:1. - - -△- - -, purified biMAb; —●—, MOV19.

all GL183$^-$ clones efficiently lysed IGROV1 cells, even at low effector:target ratios, 4 out of 5 GL183$^+$ NK-cell clones displayed only low levels of cytolytic activity against IGROV1. In the presence of NMB.45 biMAb the cytolytic activity of these GL183$^+$ clones was greatly enhanced. On the other hand, no substantial increase in cytolytic activity could be observed on GL183$^-$ clones. Analysis of CD3$^+$ TCR α/β^+ cytolytic clones showed that NMB.45 biMAb had no effect; in contrast, a biMAb formed by an anti-CD3 and an anti-ovarian carcinoma MAb (OCTR) (Mezzanzanica *et al.*, 1988) induced an efficient lysis of target cells. These data are not surprising since the CD16 molecule could not be detected by immunofluorescence at the surface of these clones. On the other hand, 1 out of 4 CD3$^+$ TCR γ/δ^+ clones expressed low levels of surface CD16 antigen and lysed IGROV1 following the addition of biMAb.

TABLE II – THE AM18.4 HYBRID (IgG_1/IgG_{2a}) BIMAb, DIRECTED TO THE TCR γ/δ AND TO OVARIAN CARCINOMA CELLS, DOES NOT MEDIATE ADCC BY NK CELLS

MAb added	Effector cells		
	PBL	NK clone 1.20.15	TCRγ/δ + clone F133
None	4[1]	10	3
MOV19 5 μg/ml	15	22	4
0.5 μg/ml	10	20	3
AM18.4 (anti-Vδ1)			
biMAb 5 μg/ml	6	12	47
0.5 μg/ml	3	12	58
NMB.45 (anti-CD16)			
biMAb 5 μg/ml	26	52	5
0.5 μg/ml	38	60	3

[1]Data are expressed as percentage of ^{51}Cr release from IGROV1 target cells. E:T ratio was 20:1 for PBL, 3:1 for the NK clone and 5:1 for the TCR γ/δ^+ clone.

DISCUSSION

In the present study we show that biMAb formed by anti-

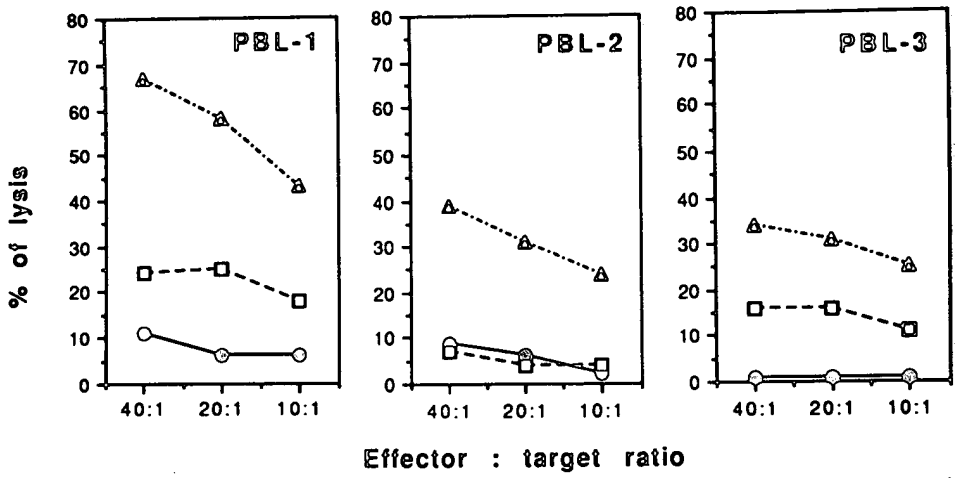

FIGURE 5 – Effect of purified biMAb (100 ng/ml) on the cytolytic activity of resting PBL isolated from 3 representative healthy donors. Target cells were IGROV1 ovarian carcinoma cells. – · –△– · –, biMAb; - - -□- - -, MOV19; —○—, no antibody.

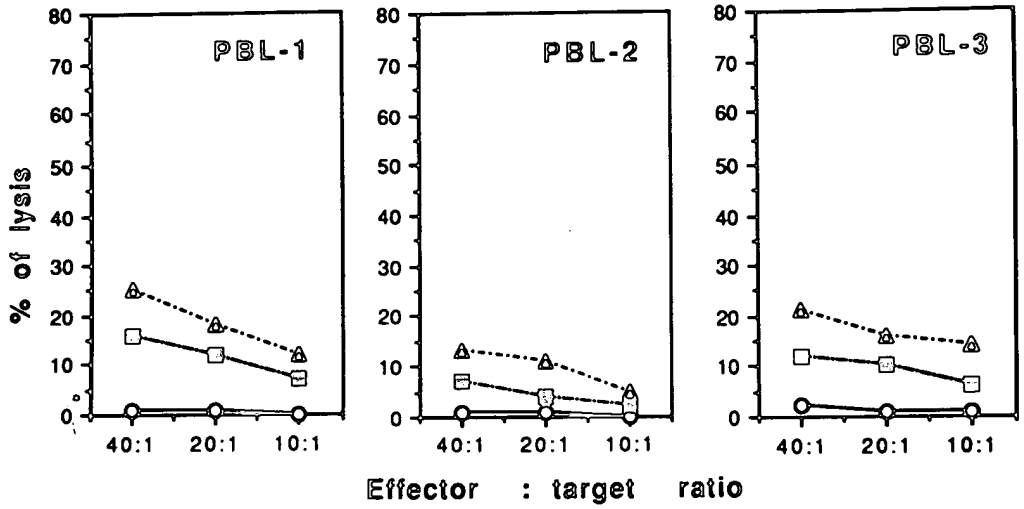

FIGURE 6 – Targeting of resting PBL against fresh MOV19$^+$ ovarian carcinoma cells by purified biMAb. – · –△– · –, biMAb; – · –□– · – MOV19; —○—, no antibody.

TABLE III – TARGETING OF DIFFERENT CLONES AGAINST OVARIAN CARCINOMA CELLS BY PURIFIED BIMAbs DIRECTED TO CD16 (NMB.45) OR CD3 (OCTR) SURFACE MOLECULES

Clone (phenotype)	% of lysis in the presence of			
	No MAb	MOV19	NMB.45 biMAb	OCTR biMAb
T1 ($CD3^-16^+GL183^+$)[1]	7[2]	36	65	n.t.[3]
T49 ($CD3^-16^+GL183^+$)	1	33	66	n.t.
T134 ($CD3^-16^+GL183^+$)	3	16	45	n.t.
C8 ($CD3^-16^+GL183^+$)	2	21	53	n.t.
C52 ($CD3^-16^+GL183^+$)	48	64	87	n.t.
F4 ($CD3^-16^+GL183^-$)	70	70	83	n.t.
F25 ($CD3^-16^+GL183^-$)	77	83	85	n.t.
F73 ($CD3^-16^+GL183^-$)	73	81	85	n.t.
TT3 ($CD3^+16^-4^-8^+WT31^+$)	0	0	0	35
S5.9 ($CD3^+16^-4^-8^+WT31^+$)	4	2	3	50
B3 ($CD3^+16^-4^-8^-BB3^+$)	27	24	30	74
M53 ($CD3^+16^+4^-8^-BB3^+$)	15	18	35	78
M68 ($CD3^+16^-4^-8^-BB3^+$)	17	20	21	75

[1]The surface phenotype of the clones was assessed by indirect immunofluorescence and FACS analysis.–[2]Data are expressed as percentage of ^{51}Cr release from IGROV1 cells at an effector:target cell ratio of 2.5:1.–[3]Not tested.

CD16 and anti-ovarian carcinoma MAbs can efficiently induce tumor-cell lysis by resting NK cells and by a subset of cloned, IL-2 cultured, NK cells.

It is well known that peripheral-blood-derived resting NK cells display cytolytic activity against a limited number of susceptible tumor-cell lines (Herberman and Ortaldo, 1981;

Trinchieri and Perussia, 1984), whereas upon activation with IL-2 they lyse most tumor cells including freshly derived ones (Phillips and Lanier, 1986; Ferrini *et al.*, 1987). The possibility of directly triggering resting NK cells to lyse "NK-resistant" tumor target cells by anti-CD16 biMAbs may have important implications. Thus, NK effector cells to be used for biMAb targeting in immunotherapy would not require the step of *in vitro* cell activation and culture. Triggering of NK cells by anti-CD16 biMAb utilizes an activation pathway which is physiologically initiated by the low-affinity Fcγ-receptor molecule (Fcγ-R III or CD16) following interaction with its natural ligand (IgG immune complexes) (Anegon *et al.*, 1988; Cassatella *et al.*, 1989). Our present data suggest that NK-cell triggering by anti-CD16 biMAb is more efficient than triggering via IgG immune complexes. Indeed, induction of target-cell lysis in the presence of biMAb was more efficient than the ADCC induced by the parental anti-target MOV19 MAb. In addition, the concentrations of biMAb capable of inducing cytolytic activity were considerably lower than the concentrations of MOV19 MAb required to obtain a cytolytic effect in ADCC. These data are likely to reflect differences in the binding affinity of Fcγ-receptor for anti-CD16 MAbs or for the Fc portion of IgG.

That induction of cytolysis by the hybrid NMB.45 biMAb molecule (IgG_1/IgG_{2a}) was not mediated by the interaction of its Fc receptor with CD16 was indicated by the lack of triggering effect on $CD16^+$ effector cells of another IgG_1/IgG_{2a} biMAb. This biMAb was directed against the same tumor-associated antigen and against a determinant of the TCR γ/δ which is not expressed on $CD16^+$ effector cells. In agreement with our present results on murine hybrid antibodies, previous studies showed that rat IgG2b MAbs, but not hybrid IgG_{2b}/IgG_{2c} biMAbs, mediated ADCC by human $CD16^+$ effector cells (Clark and Waldmann, 1987).

Our study confirms and extends previous reports showing that hetero-cross-linked antibodies produced by chemical procedures can trigger tumor-cell lysis by NK cells via CD16 (Titus *et al.*, 1987; Garrido *et al.*, 1990) or CD2 (Scott *et al.*, 1989). Our data, based on the use of a "native" biMAb produced by a hybrid hybridoma, indicate that monovalent anti-CD16/anti-tumor biMAbs are also effective. In addition, we showed that among IL-2-induced, cloned, $CD3^-16^+$ NK cells, heterogeneity exists in the susceptibility to triggering by anti-CD16 biMAbs. In this context, 2 phenotypically stable subsets of NK cells have been recently defined on the basis of the surface expression of a molecule (GL183) involved in NK-cell activation and function (Moretta *et al.*, 1990). While $GL183^-$ clones expressed high levels of cytolytic activity against ovarian carcinoma cells, which could not be further enhanced by the addition of biMAb, the low levels of cytolytic activity mediated by $GL183^+$ clones could be greatly increased by biMAb. This indicates that, although $GL183^+$ NK clones are equipped with an efficient lytic machinery, the interaction with tumor target cells does not provide an adequate signal to trigger cytolytic function.

Evidence has been provided for heterogeneity in the expression of the CD16 molecule on the TCR γ/δ^+ subset (Groh *et al.*, 1989). Our present data confirm, at the clonal level, that such heterogeneity does exist. Thus, only 1 out of 4 TCR γ/δ^+ clones expressed low levels of CD16 molecule and could be triggered by anti-CD16 biMAb. On the other hand, all clones were efficiently triggered by an anti-CD3 biMAb with the same anti-tumor specificity.

In conclusion, our data indicate that anti-CD16 biMAb may represent a suitable reagent for the selective targeting of NK cells against tumors.

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